Loss of flavonoid pigments with ageing in male *Polyommatus icarus* butterflies (Lycaenidae)

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Summary. *Polyommatus icarus* (Rottemburg, 1775) butterflies contain UV-absorbing flavonoid pigments sequestered from their larval hostplants. A comparison of freshly emerged laboratory-bred males with field-caught individuals of increasing wing wear, as a measure of age, revealed that the butterflies lose a substantial fraction of their flavonoid load, via loss of scales, during the first few days of their adult life-span. Possible consequences for intraspecific visual communications are discussed. Our findings show that the physiological status of butterflies, in addition to larval diet and sex, contributes to the pronounced intraspecific variation of flavonoid load, and thus UV wing pattern, in *P. icarus*.

Zusammenfassung. Falter von *Polyommatus icarus* (Rottemburg, 1775) enthalten aus der larvalen Nahrung sequestrierte Flavonoide als UV-absorbierende Pigmente. Ein Vergleich gezüchteter, frisch geschlüpfter Männchen mit Freilandfängen unterschiedlichen Alters und Abnutzungsgrades der Flügel zeigt, daß die Schmetterlinge schon innerhalb der ersten Tage ihrer adulten Lebensspanne einen Großteil der Flavonoide durch Schuppenverluste einbüßen. Mögliche Konsequenzen für die innerartliche visuelle Kommunikation werden angesprochen. Unsere Beobachtungen zeigen, daß neben der larvalen Nahrung und dem Geschlecht auch der physiologische Status der Tiere zur ausgeprägten innerartlichen Variabilität der Flavonoidbeladung und damit des UV-Flügelmusters von *P. icarus* beitragen.

Résumé. Les adultes de *Polyommatus icarus* (Rottemburg, 1775) contiennent des pigments flavonoïdes absorbant la radiation UV, séquestrés de leurs plantes nourricières larvaires. La comparaison de mâles de culture en laboratoire fraichement éclos à des individus ramassés dans la nature et frottés à divers degrés progressifs, en mesure de leur âge, à révélé que les papillons perdent une proportion substancielle de leur contenu en flavonoïdes, par la perte d'écailles, durant les premiers jours de leur vie adulte. Les implications possibles au niveau de leur communication visuelle intraspécifique sont discutées. Nos observations démontrent que l'état physiologique des papillons, complémentairement à leur nutrition à l'état larvaire et au sexe, contribue à la variation intraspécifique prononcée du contenu en flavonoïdes, et par conséquent à celle de la réflection alaire des rayons UV, chez *P. icarus*.

Key words: Lycaenidae, *Polyommatus icarus*, flavonoid pigments, ageing, visual communications.

Introduction

Wing colour patterns frequently play an important role in visual communication among butterflies. Differences between species in wing patterns may facilitate reproductive isolation, although many butterflies still recognize conspecifics even after major experimental manipulation (Silberglied 1984), suggesting that the importance of wing pattern differences for species recognition has frequently been overestimated. More often, intraspecific variation in brightness and intensity of colouration may serve as signal during mate choice or male-male interactions (Silberglied 1984; Brunton &

Majerus 1995). According to these authors conspicuous colour patterns of (usually) male butterflies (mimicry and aposematism being set aside) have largely evolved through intrasexual selection, although Darwinian intersexual selection (Smith 1984) and more complex interactions may also have been involved (Vane-Wright 1984; Vane-Wright & Boppré 1993).

Butterfly wing patterns are determined by two types of colours: structural colours due to interference and diffraction within specialized scales, and colours which result from absorption of specific spectral fractions of the light by pigments contained in the scales (Nijhout 1991). Since the eyes of most butterflies studied thus far are sensitive from the human-visible spectrum far into the UV-range (sensitivity typically stretches from 340 to 700 nm: Eguchi et al. 1982; Bernard & Remington 1991), any studies of biological functions of butterfly wing patterns must consider this entire spectral range (Silberglied 1984; Endler 1990).

Brigthness and intensity of UV reflection patterns can decrease with ageing due to increasing wing wear (i.e. loss of scales) and may then affect mating opportunities (Rutowski 1985). We here report on a special case of such age-related changes. Numerous butterflies sequester flavonoids from their larval hostplants, and during metamorphosis incorporate these plant-derived pigments into their wings (Ford 1941; Nijhout 1991). Flavonoids may have various colours to the human eye (often yellow), but they all strongly absorb UV-light with two distinct maxima. One of these maxima lies in the range of 240–280 nm and is invisible to insects, but the other maximum is situated, depending on the specific chemical compound, in the range between 300–380 nm (Harborne 1991) and thus visible to UV-sensitive insect compound eyes.

Recent work on the sequestration of flavonoids by lycaenid butterflies has shown that (1) only particular hostplant flavonoids are stored by the larvae, while others are excreted; (2) lycaenid larvae are capable of metabolizing flavonoids, usually by means of glycosylation; (3) 60–80% of an adult butterfly's flavonoid load is situated in the scales of the ventral wing surfaces; and (4) females typically sequester larger amounts and concentrations of these phenolic pigments than males (Wiesen et al. 1994; Burghardt et al. 1997; Geuder et al. 1997; Schittko et al. 1999). As a result, adult butterflies of the common blue butterfly, *Polyommatus icarus* (Rottemburg 1775), for example, strongly vary with regard to their flavonoid content, depending on sex and hostplant used during the larval stages. This variation translates into differences in underside wing patterns in the UV-range (Knüttel & Fiedler 1999), and at least for females such variation is biologically significant. Females richer in flavonoids are more attractive to mate-searching males (Burghardt et al. 2000).

Against this background we set out to investigate how flavonoid load of adult butterflies changes with age and wingwear. Specifically we tested the hypothesis that flavonoid content of older, worn individuals would decrease due to the loss of flavonoid containing scales.

Material and Methods

Butterflies of the summer generation were collected at various locations in northern Bavaria (around Würzburg, Bayreuth, and Regensburg). Since males are more easily found in the field, and in order to minimize damage to natural populations, only this sex was sampled for the present study. Butterflies were killed immediately after capture, frozen, freeze-dried and stored at −20°C until needed for chemical analyses. Special care was taken not to inflict any damage to the specimens apart from their natural wingwear. To obtain butterflies with complete flavonoid load prior to any scale losses, we took males from a laboratory culture, whose parental stock also had originated from Würzburg. These butterflies had been raised in an environmental chamber at 22.5°C (see Burghardt & Fiedler 1996 for details), using flowers of *Lotus corniculatus* L. (a preferred hostplant of northern Bavarian populations: Ebert & Rennwald 1991, own field observations) as larval food.

Before analysis each butterfly was weighed to the nearest 0.1 mg (on an electronic balance), its forewing length measured to the nearest 0.1 mm (using precision calipers), and its wing wear was subjectively classified into one of the following four categories:

I. Fresh, totally undamaged butterfly (here only reared males which were killed 1–2 h after eclosion);

II. Light wing wear, loss of scales discernible (e.g. at fringes), but colour still brilliant blue (to the human eye);

III. More severe wing wear, loss of scales distinct, but less than 5% of wing area missing, blue upperside colour fading;

IV. Severely worn, pronounced scale losses all over, larger fractions of wing surface damaged (e.g. along margins), colouration dull.

For chemical analyses of the flavonoid content, each specimen was individually ground in a mortar and extracted in 90% methanol (MeOH) + 10% H_2O. To exhaustively recover soluble flavonoids, extraction was repeated three times over 24 h each under permanent stirring. The total extract was then filtered, particles removed using a centrifuge, and analysed by means of HPLC (GynkotekM480, equipped with column heater, temperature set at 20°C; column: Eurospher 100-C18, 125 × 4 mm) using a linear $MeOH/H_3PO_4$ gradient (starting with 10%MeOH + 90% H_3PO_4, after 50 min: 100%MeOH, flow rate: 1ml/min; see Schittko et al. 1999 for further details).

Flavonoids were detected by UV-VIS spectroscopy (using a UVD 340S detector). As far as possible, flavonoid compounds were identified using a spectral library stored in the computer system of the HPLC equipment, or by comparison with flavonoids previously isolated and identified from larval hostplants (Burghardt 2000). However, for the purpose of the present study the total flavonoid load of butterflies was more important than identification of each substance, since all flavonoids contribute to the UV-wing pattern due to their absorption properties. To assess this complete flavonoid load as accurately as possible, all peaks in each HPLC chromatogram were individually checked for their characteristic bimodal UV spectrum, and each peak which showed UV absorption in both ranges characteristic for flavonoids (240–280 nm due to benzoyl ring, 300–

380 nm due to cinnamoyl double ring) was scored as a flavonoid. Quantification of flavonoid peaks was made possible by adding a known amount of one flavonoid (Kaempferol, Roth™, purity >98%) as internal standard to each extract prior to HPLC analysis. Free unglycosylated Kaempferol does not occur in *P. icarus* butterflies (Burghardt 2000) and hence does not affect the results. Calibrated by this internal standard, the area of each peak in a chromatogram could then be converted into a measure of amount, or concentration, of the respective compound. The total sum of all these compounds is subsequently referred to as 'flavonoid load', and the ratio of flavonoid load versus dry mass is referred to as 'flavonoid concentration'. Data were evaluated statistically using StatSoft (1999). Throughout the text, mean values are given ±1SE.

Results

Freshly emerged male butterflies reared on *L. corniculatus* flowers were rather rich in flavonoids (26.48±1.60μg, range: 16.33–36.87μg), but even with slight wingwear flavonoid load decreased drastically (8.10±1.22μg; range: 2.54–15.67μg) and further diminished with increasing wingwear. Two totally worn males contained less than 2μg flavonoids per individual. These differences were highly significant (Kruskal-Wallis test: $H_{3df} = 33.10$, p<0.0001). When controlling for body mass, the pattern with regard to flavonoid concentrations remained unchanged (Fig. 1).

Reared, freshly eclosed males were much richer in flavonoids than even moderately worn, field caught butterflies, despite the fact that the latter were significantly larger (fore wing length, field caught: 15.97±0.15 mm; reared: 14.56±0.15mm; t-test, p<0.01). No significant differences, however, occurred with regard to dry mass (field caught: 10.03±0.32 mg; reared: 9.36±0.35mg; t-test, p>0.10). Within neither of the wingwear classes I, II, and III (where enough individuals were available to allow for quantitative analysis) was there any significant (positive or negative) correlation between butterfly size (measured either as fore wing length or dry mass) and flavonoid richness (measured as total flavonoid load or concentration, respectively: Pearson correlations, p>0.07 in each case).

Discussion

Our results show that, as expected, *P. icarus* butterflies lose UV-absorbing flavonoid pigments in the course of ageing. Progressive loss of pigmented scales is certainly the only significant mechanism repsonsible for such flavonoid losses. After metamorphosis no metabolism takes place within pigmented scales, and flavonoids are so robust against other means of degradation (e.g. photo-oxidation) that during the short lifespan of an adult *P. icarus* butterfly such effects are negligible. Age-related changes in the flavonoid load of butterflies have a couple of ramifications. First, it may be inappropriate to quantitatively estimate flavonoid load of field-caught specimens without accounting for butterfly age and wing wear. Not only is it problematic to draw conclusions in polyphagous species, when the larval feeding history of a specimen remains unknown (as for example in Wilson (1987), where hostplant relationships were in-
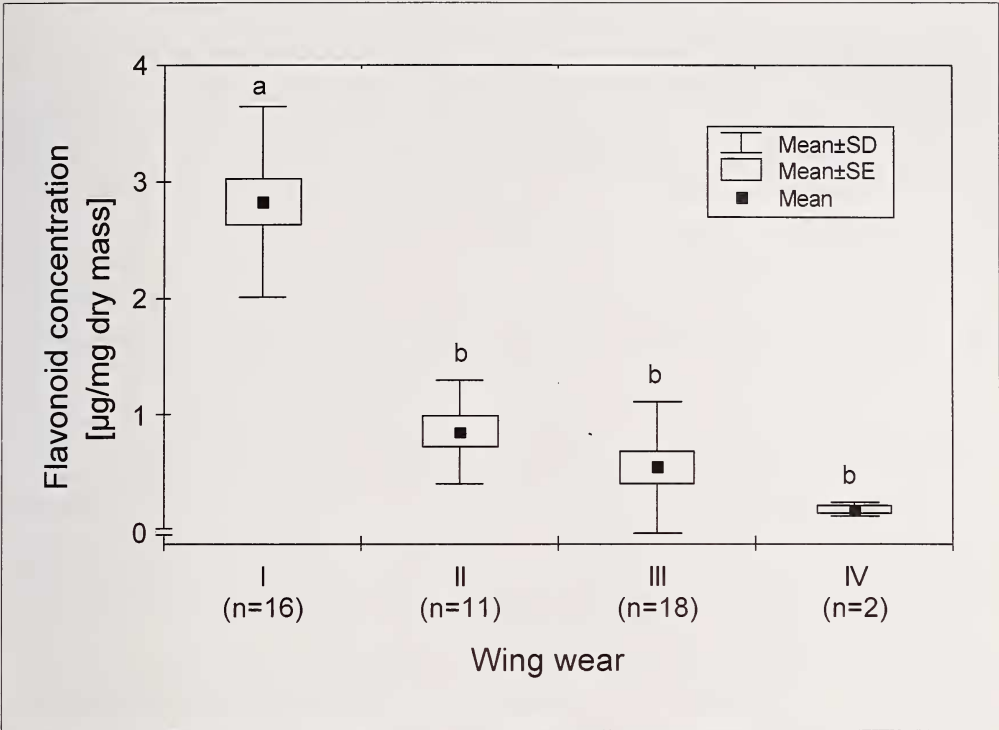
Fig. 1. Decrease of total flavonoid concentration [µg/mg dry mass] in individual males of *Polyommatus icarus* with increasing wing wear. I: undamaged, freshly eclosed male; to IV: strongly worn, tattered male (for definition of categories see text). Overall differences are highly significant (Kruskal-Wallis test: H_{3df} = 33.94, p<0.0001). Boxes marked with the same letter do not differ significantly (Tukey-Kramer test, significance level set as p=0.05).

ferred from – partly even erroneous – literature data). In addition to this qualitative source of uncertainty, butterflies apparently quickly lose a substantial fraction of their original flavonoid load contained in wing scales in the course of flight activity. Judging from own extensive field experience and mark-release-recapture studies, *P. icarus* males in the wing wear class II were probably just 1–3d old, and those in class III about 4–8d (Tillmanns 1995; Fiedler, unpublished observations).

Our representatives of wing wear class I (young, freshly emerged) had all been bred in the laboratory on a very favourable, flavonoid-rich diet (Burghardt 2000). We cannot be sure that all, or at least a majority of, our field-caught specimens had really fed on *L. corniculatus* as larvae, although during a detailed field study conducted at the campus of Würzburg university more than 70% of all observed ovipositions occurred on this plant species (Tillmanns 1995). In fact, the flavonoid profile of one male specimen (from Volkach near Würzburg) strongly suggests the larval hostplant in this case was *Medicago sativa* L. However, if raised on *M. sativa* inflorescences, flavonoid load of *P. icarus* butterflies is even higher than when fed *L. corniculatus* (Burghardt 2000). In a comparison among butterflies reared on six naturally used hostplants, flavonoid loads

and concentrations of specimens from *L. corniculatus* always scored at an average rank (Burghardt 2000). Hence, the choice of butterflies reared on this particular hostplant for comparison with field-caught individuals should not have exaggerated our results.

Second, wing wear in *P. icarus* males is not only reflected in a fading of the brilliant iridiscent coulour of the dorsal wing surfaces (which is brought about by structural colours and extends far into the UV-range), but also affects the absorption pattern of the ventral wing surfaces, and here notably the UV-absorption through flavonoids. For the visual system of insects capable of UV-vision, UV-absorbing pigments superimposed on a colour pattern in other parts of the spectral range can enhance colour saturation and thus visibility (e.g. Lunau 1992). This means that a fresh butterfly with intact reflectance colouration on the dorsal surface plus intensive and more saturated underside colour pattern should be more conspicuous to conspecifics in the field, for example over larger distances. We do not know yet whether in *P. icarus* intraspecific colour differences play a role in male-male interactions, or increase attractiveness of males for females during courtship. However, male-male combats are very common during patrolling flights of *P. icarus* (Lundgren 1977), and it is well conceivable that wing colouration and wingwear serve as signals in this context (cf. Brunton & Majerus 1995).

Third, although our data shown above refer to male butterflies, females most likely experience similar losses of flavonoids (and of course other pigments) with progressive scale loss when ageing. In *P. icarus* (Wiesen et al. 1994; Burghardt et al. 1997; Schittko et al. 1999), as well as in the related *P. bellargus* (Rottemburg, 1775), females even sequester distinctly more flavonoids than males. Hence, the effect of these pigments on UV wing patterns is more pronounced in females, and consequently one should expect an even stronger difference between flavonoid-rich young individuals and older, worn females. For females sitting in the vegetation and waiting for mate-searching males which patrol through the habitat, richness in flavonoids substantially increases their attractiveness to the opposite sex (Burghardt et al. 2000). Thus, the older and more worn a female, the more may her chances decrease to be located by a male. A high flavonoid load, which renders a female more conspicuous to potential mates, could therefore assist in reducing her risk of pre-reproductive mortality (cf. Zonneveld & Metz 1991). Further experiments are required to assess whether among lycaenid butterflies richness in plant-derived flavonoid pigments signals 'quality' or 'youth' to potential mates (i.e. directly reflects some fitness components: Møller & Alatalo 1999), or rather just enhances visibility (i.e. provides superior stimulation of the visual capacities of conspecifics, cf. Ryan & Keddy-Hector (1992) for the reverse situation where females choose among competing males).

Our findings presented here demonstrate that interactions between lycaenid butterflies and flavonoids in their hostplants are even more complicated than previously thought. Apart from larval food and gender, the physiological status of a butterfly must also be considered as additional source of phenotypic variation in pigment load and thus UV wing pattern.

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